Exhibit 21

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5-Aminolevulinic Acid, but not 5-Aminolevulinic Acid Esters, is Transported into Adenocarcinoma Cells by System BETA Transporters

Eva Rud[1], Odrun Gederaas[1,2], Anders Høgset[1] and Kristian Berg[1,*]

[1]Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway
[2]Department of Clinical Chemistry, University Hospital, Trondheim, Norway

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ABSTRACT

5-aminolevulinic acid (5-ALA) and its ester derivatives are used in photodynamic therapy as precursors for the formation of photosensitizers. This study relates to the mechanisms by which 5-ALA is transported into cells. The transport of 5-ALA has been studied in a human adenocarcinoma cell line (WiDr) by means of [^{14}C]-labeled 5-ALA. The rate of uptake was saturable following Michaelis–Menten kinetics (K_m = 8–10 m*M* and V_{max} = 18–20 nmol·(mg protein × h)$^{-1}$), and Arrhenius plot of the temperature-dependent uptake of 5-ALA was characterized by a single discontinuity at 32°C. The activation energy was 112 kJ·mol^{-1} in the temperature range 15°–32°C and 26 kJ·mol^{-1} above 32°C. Transport of 5-ALA was Na^+ and partly Cl^--dependent. Stoichiometric analysis revealed a Na^+:5-ALA coupling ratio of 3:1. With the exception of valine, methionine and threonine, zwitterionic and basic amino acids inhibited the transport of 5-ALA. 5-ALA methyl ester was not an inhibitor of 5-ALA uptake. The transport was most efficiently inhibited, *i.e.* by 65–75%, by the β-amino acids, β-alanine and taurine and by γ-aminobutyric acid (GABA). Accordingly, 5-ALA, but not 5-ALA methyl ester, was found to inhibit cellular uptake of [^{3}H]-GABA and [^{14}C]-β-alanine. Protoporphyrin IX (PpIX) accumulation in the presence of 5-ALA (0.3 m*M*) was attenuated 85% in the presence of 10 m*M* β-alanine, while PpIX formation in cells treated with 5-ALA methyl ester (0.3 m*M*) or 5-ALA hexyl ester (4 μ*M*) was not significantly influenced by β-alanine. Thus, 5-ALA, but not 5-ALA esters, is transported by β-amino acid and GABA carriers in this cell line.

INTRODUCTION

Topical or systemic administration of 5-aminolevulinic acid (5-ALA)†, as used in photodynamic therapy (PDT), results in accumulation of porphyrins, in particular, protoporphyrin IX (PpIX) (1,2). The initial step in the heme synthesis pathway is the 5-ALA synthase–induced formation of 5-ALA from succinyl-CoA and glycine, and this step is regulated by feedback inhibition by heme (3). By treating cells with 5-ALA, this negative feedback can be overruled. Due to the hydrophilic properties of ALA, ALA-PDT may clinically be limited by the rate of ALA uptake into the neoplastic cells and/or its penetration through the tissue (55,56). Clinical work has shown that more than 90% of superficial basal cell carcinomas (BCC) respond well to the topically applied ALA-based PDT. But, there is a low complete response rate to this treatment for the nodular BCC which comprises 45–60% of all the BCC. 5-ALA esters with increased lipophilicity have therefore been established in order to increase the penetration depth of the precursor and efficacy of 5-ALA in PDT. Preclinical studies indicate that 5-ALA esters induce PpIX more efficiently after topic applications than 5-ALA (57) and phase-III clinical trials with 5-ALA methyl ester for BCC have been initiated.

The initial step in the 5-ALA–induced synthesis of porphyrins is the penetration of 5-ALA through the plasma membrane. This step may be a rate-limiting factor in the formation of PpIX. This is also in accordance with the observation that esterification of 5-ALA with aliphatic alcohols (C_6 or longer) was found to reduce 30–150-fold the amount of drug needed to reach the same level of PpIX accumulation (4). Such observations indicate that long-chain 5-ALA esters are taken up more efficiently and through other pathways than 5-ALA. However, the mechanisms associated with the uptake of 5-ALA and 5-ALA esters in neoplastic cells are not known. This prompted us to study the plasma membrane transport of 5-ALA in a human adenocarcinoma cell line (WiDr). It is shown in this report that 5-ALA, but not 5-ALA esters, is taken up by carrier systems transporting the β-amino acids, β-alanine and taurine, as well as γ-aminobutyric acid (GABA) in these cells.

MATERIALS AND METHODS

Cell cultivation. Cells of an established line (WiDr), derived from a human primary adenocarcinoma of the rectosigmoid colon (5), were subcultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY) containing 10% fetal calf serum, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin and 2 m*M* L-glutamine. The cells were subcultured approximately twice a week

*To whom correspondence should be addressed at: Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. Fax: 47-22-93-42-70; e-mail: kristian.berg@labmed.uio.no

†*Abbreviations:* 5-ALA, 5-aminolevulinic acid; BCC, basal cell carcinomas; GABA, γ-aminobutyric acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid; PBS, Dulbecco's phosphate-buffered saline; PDT, photodynamic therapy; PpIX, protoporphyrin IX; RPMI, Roswell Park Memorial Institute.

(split ratio, 1:100) and maintained at 37°C and 5% CO_2 in a humid environment.

Chemicals. δ-[4-^{14}C]-ALA (specific activity 47.6 mCi·mmol^{-1}) was purchased from NEN™ Life Science Products (Boston, MA). Unlabeled 5-ALA (Sigma, St. Louis, MO or Photocure, Norway) was dissolved in Dulbecco's phosphate-buffered saline (PBS), RPMI 1640 medium without serum, or a buffer containing 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES), 150 m*M* NaCl, 1.2 m*M* $CaCl_2$, 0.64 m*M* $MgCl_2$, 6.0 m*M* KOH and 5.0 m*M* D-glucose (named HEPES-buffer). pH was adjusted to 7.4 with 5 *M* NaOH. 5-[^{14}C]ALA was diluted 48-fold with this solution of cold 5-ALA. [2,3-^{3}H(N)]-GABA was obtained from NEN™ Life Science Products, while [1-^{14}C]-β-alanine was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals were of analytical grade and of highest purity commercially available. All amino acids used were L-stereoisomers.

Uptake measurements. The cells (2.5–5 × 10^4 cells·cm^{-2}) were seeded into 24-well plates 2–3 days before the experiments; nearly confluent layers of cells were incubated with radiolabeled material at the desired concentration under various conditions. The cells were either incubated in RPMI 1640 medium without serum, PBS or in the HEPES-buffer. After treatment, the cells were kept on ice and washed four times in ice-cold PBS. The cells were dissolved in 200 μL 0.1 *M* NaOH. After 10 min of incubation 3 mL scintillation fluid (Opti-fluor, Packard, Menden, USA) was added to the samples, and radioactivity was measured in a Packard Tri-Carb 4550 scintillation counter.

Measurement of cellular protoporphyrin IX content. Cells (2.5–5 × 10^4 cells·cm^{-2}) were seeded into six-well plates 2–3 days before experiments and treated as described above. After treatment the cells were washed twice in ice-cold PBS and brought into a solution of 1.0 *M* $HClO_4$ in 50% methanol by scraping with a Costar cell scraper. Cell debris was removed by centrifugation. PpIX was quantitatively extracted from the cells by this procedure (6). The PpIX content of the samples was detected spectrofluorometrically using a Perkin–Elmer LS50B spectrofluorometer. PpIX was excited at 408 nm and fluorescence measured at 605 nm. A long-pass cut-off filter (530 nm) was used on the emission side. A standard of known concentration was added to the samples at a concentration increasing the total fluorescence by approximately 50%.

Protein determination. Protein was assayed by Bradford's method (7) using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) with human serum albumin as the standard.

Statistical analysis. Uptake measurements were routinely done in duplicate. Each experiment was repeated two or three times. The results are presented as means ± standard error of measurement. Linear regression analysis was performed by the method of least squares.

RESULTS

5-Aminolevulinic acid uptake kinetics

5-ALA that has entered the cytoplasm may enter the heme synthesis pathway. In this study radioactively labeled 5-ALA has been utilized for measuring cellular uptake of 5-ALA. Some of the counts registered by the scintillation counter may thus stem from heme intermediates. However, this does not influence the results since the uptake rates were independent of the presence of succinyl acetone, an inhibitor of the enzyme 5-ALA dehydratase, which converts 5-ALA into porphobilinogen (8).

Figure 1a shows the time-course of 5-ALA uptake at different concentrations of 5-ALA. At all concentrations (0.1–10 m*M*) uptake of 5-ALA was linear during the first 5 h. Cytotoxic effects were observed when the cells were treated for more than 3 h with 20 m*M* 5-ALA. The rate of 5-ALA uptake (Fig. 1a) was plotted as a function of 5-ALA concentration (Fig. 1b). These results were fitted by regression analysis to the function y = ax/(b + x) according to the Michaelis–Menten function, $v = V_{max}[S]/(K_m + [S])$, in which v is the rate of 5-ALA uptake, V_{max} the maximum rate of 5-ALA uptake, [S] the concentration of 5-ALA, and K_m the concentration of 5-ALA inducing ½ × V_{max}. The rate of 5-ALA uptake was well fitted to the above function (r^2 = 0.99) with K_m = 8.4 m*M* and V_{max} = 18 ± 0.9 nmol·(mg protein × h)$^{-1}$. Essentially identical results were obtained when calculating and plotting the data according to the Eadie–Hofstee equation, K_m = 10 ± 1.6 m*M* and V_{max} = 20.7 ± 2.5 nmol·(mg protein × h)$^{-1}$ (Fig. 1c).

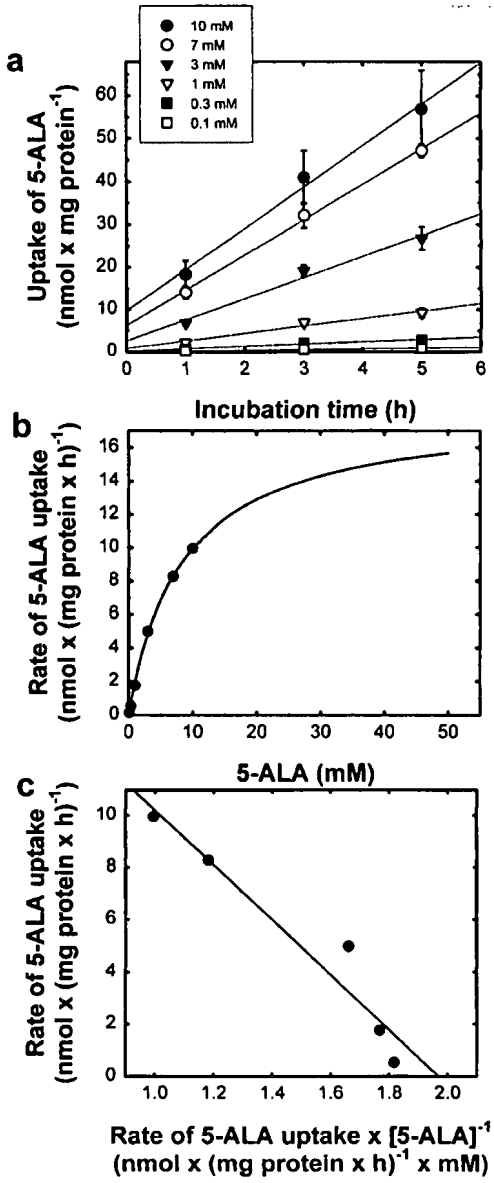

Figure 1. Time course and kinetics of 5-ALA transport in WiDr cells. Uptake of 5-ALA was measured in RPMI 1640 medium over a concentration range of 0.1–10 m*M* as indicated in (a) and analyzed for cellbound 5-ALA as described in the Experimental section. The linear regression lines obtained in (a) were used to plot the rate of 5-ALA uptake *vs.* concentration of extracellular 5-ALA (b) and an Eadie–Hofstee plot (v/s *vs.* v).

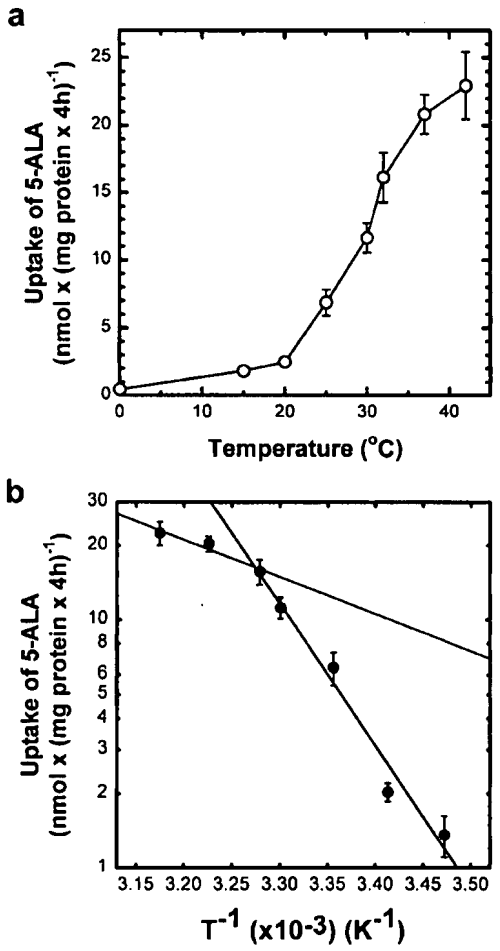

Figure 2. Effect of temperature upon 5-ALA transport. WiDr cells were incubated for 4 h in PBS containing 1 m*M* 5-ALA and 5 m*M* glucose at the indicated temperatures and otherwise treated as described in Materials and Methods (a). The results are also presented in an Arrhenius plot (b). Linear regression lines were obtained by the method of least-squares (r^2 = 0.977 and r^2 = 0.941 in the regions 15–32°C and 32–42°C, respectively). In the Arrhenius plot, binding of 5-ALA to the plasma membrane (at 0°C) was subtracted in order to assess only the uptake of 5-ALA.

Temperature-dependence of 5-aminolevulinic acid uptake

The influence of incubation temperature upon the uptake kinetics of 5-ALA was evaluated after 4 h of incubation with 1 m*M* 5-ALA in PBS containing 5 m*M* D-glucose (Fig. 2a). The rate of 5-ALA uptake was found to be clearly temperature-dependent, with negligible uptake at 0°C. The small fraction of 5-ALA associated with the cells at 0°C did not increase with time of incubation, and is therefore most likely due to 5-ALA associated with the plasma membrane. The activation energy for 5-ALA uptake was calculated by linear least squares regression analysis of a plot of log k against $1/T$ according to the Arrhenius equation (Fig. 2b):

$$\log k = \log A = \frac{E_a}{2.303RT},$$

in which k is the uptake of 5-ALA, A the frequency factor and E_a the activation energy. The Arrhenius plot revealed a change in slope at 32°C with activation energies of E_a = 112 kJ·mol^{-1} below 32°C and E_a = 26 kJ·mol^{-1} above 32°C (Fig. 2b).

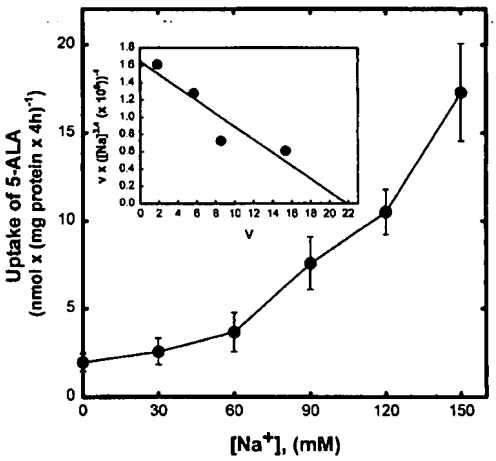

Figure 3. Dependence of 5-ALA uptake upon Na^+-concentration. WiDr cells were incubated with 1 m*M* 5-ALA in a HEPES-buffer in which the Na^+ concentration is varied by substituting NaCl with choline chloride and otherwise treated as described in Materials and Methods. Uptake of 1 m*M* 5-ALA was measured after a 3 h incubation. Inset: Hill-type plot in which the velocity (v, nmol·(mg protein × 3 h)$^{-1}$) was plotted against $v \cdot [Na^+]^{-n}$, with n = 3.4, r^2 = 0.84.

Na^+-dependence of 5-ALA uptake

The Na^+-dependence of 5-ALA uptake in WiDr cells was analyzed by substituting NaCl with choline chloride (Fig. 3). Incubation with 150 m*M* choline chloride for 3 h did not induce cytotoxic effects in the cells as revealed by microscopic inspection and propidium iodine staining. However, after 4 h of incubation a few cells started to round up. Uptake of 5-ALA in WiDr cells was, therefore, analyzed after 3 h of incubation. As seen in Fig. 3, 5-ALA uptake in WiDr cells is highly Na^+-dependent and is reduced nine-fold in the absence of Na^+. Under these conditions about 2 nmol·(mg protein)$^{-1}$ 5-ALA was associated with the cells after 3 h of incubation with 1 m*M* 5-ALA. This is a somewhat larger amount than what is expected to be surface-bound, indicating a minor Na^+-independent pathway. Measurements of PpIX formation in cells treated with 1 m*M* 5-ALA showed that PpIX formation was reduced by more than 60% in the absence of Na^+ (data not shown).

The relationship between the uptake and the Na^+ concentration was found to be nonlinear, indicating participation of more than one Na^+ per transport of one molecule of 5-ALA (Fig. 3). To calculate the number of Na ions involved per transport cycle, the experimental data over the Na^+ concentration range of 60–150 m*M* were analyzed by a Hill-type plot (v *vs.* v/[S]^n), as shown in the inset of Fig. 3. The plot gave a straight line with the best fit (r^2 = 0.84) when the Hill coefficient (n) was 3.4, suggesting that three Na ions are associated with the transport of one 5-ALA molecule.

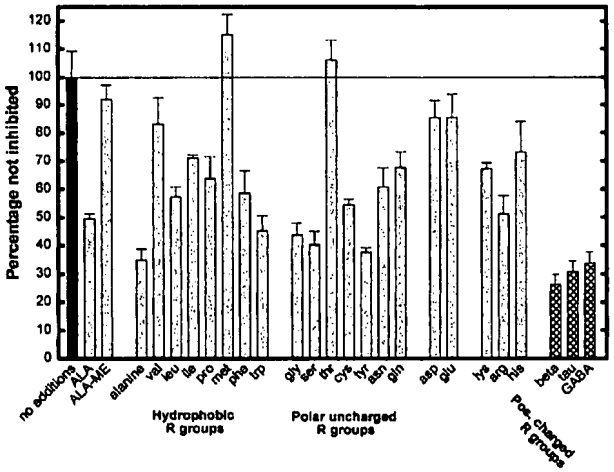

Figure 4. Inhibition of 5-ALA uptake by various amino acids. The average amount of 5-[^{14}C]ALA (23 μM) taken up by WiDr cells after 3 h in the presence of various amino acids (10 mM) including β-alanine (beta), taurine (tau) and GABA, is reported as the percentage of the mean uptake in the absence of any added nonradioactive amino acid as indicated in the figure. The cells were incubated in an HEPES-buffer as described in the Materials and Methods section.

Inhibition of 5-aminolevulinic acid uptake with α-amino acids

As seen by comparing results in Fig. 1 with those in Figs. 2 and 3, the rate of 5-ALA uptake seems to be about two-fold higher in simple salt solutions than in the amino acid-containing RPMI 1640 medium. In order to evaluate the possible uptake of 5-ALA through amino acid transporters, WiDr cells were incubated with 23 μM 5-[^{14}C]ALA and 10 mM of various amino acids, including 5-ALA and 5-ALA methyl ester (Fig. 4). It was found that nonlabeled 5-ALA inhibited the uptake of 5-[^{14}C]ALA as expected for an active transport pathway. On the contrary, 5-ALA methyl ester did not inhibit the uptake of 5-[^{14}C]ALA. The transport of 5-ALA was inhibited by basic amino acids and—with the exception of threonine, valine and methionine—all the zwitterionic α-amino acids. The acidic amino acids aspartate and glutamate did not significantly attenuate the transport of 5-ALA ($P > 0.1$).

Uptake through system BETA

5-ALA is a δ-amino acid with structural similarities to the β-amino acids, β-alanine and taurine, as well as GABA in that they all have amino and acid groups on opposite terminals of the molecule (Fig. 5). It was found that all three amino acids inhibited 5-[^{14}C]ALA uptake more efficiently than 5-ALA itself, and equally or more efficiently than all the other amino acids investigated (Fig. 4). The K_m for uptake of β-alanine and GABA through the system-BETA superfamily of transporters are usually below 100 μM (9). In accordance with this it was found that β-alanine and GABA, and the specific inhibitor of β-alanine and GABA transport—hypotaurine, fully inhibited the 5-ALA uptake at concentrations less than 300 μM (Fig. 6). Furthermore, the cellular uptake of β-alanine and GABA was clearly inhibited by 5-ALA, but not by 5-ALA methyl ester (Fig. 7).

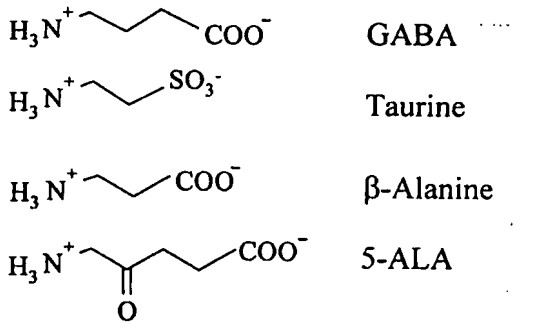

Figure 5. Some compounds structurally related to 5-ALA and employed in the study.

The system BETA superfamily of transporters are characterized by their chloride dependency (10,11). Chloride ions (Cl^-) were, therefore, substituted with a mixture of gluconate, NO_3^- and SO_3^- salts (10), and the results clearly show that the 5-ALA uptake is Cl^--dependent (Fig. 8). There appears to be a linear relationship between the [Cl^-] and the 5-ALA uptake ($r^2 = 0.994$), indicating a 1:1 Cl^-:5-ALA coupling ratio. Similar results were obtained when Cl^- was substituted with gluconate alone (data not shown). It should however be noted that 5-ALA is delivered as 5-ALA·HCl, and when all Cl^- is removed from the medium there will still be about 1 mM Cl^- left in the medium.

The present results indicate that 5-ALA is, to a large extent, taken up by WiDr cells through β-amino acid and GABA transporters. To investigate the importance of these transporters upon the rate of PpIX formation, cells were incubated with 0.3 mM 5-ALA in the presence or absence of 10 mM β-alanine (Fig. 9). In these experiments β-alanine was found to reduce the 5-ALA–induced PpIX formation by 85%. On the other hand, β-alanine had no significant effect on the PpIX formation in 5-ALA methyl ester or 5-ALA hexyl ester–treated cells.

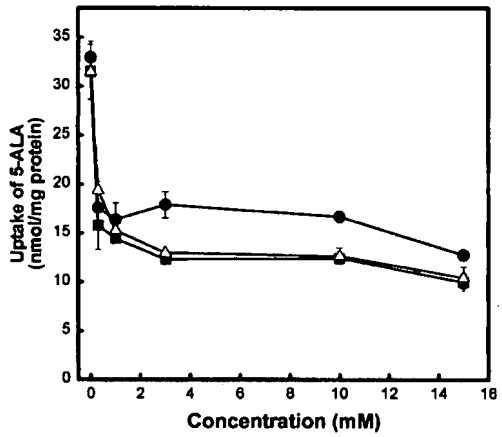

Figure 6. Inhibition of 5-ALA uptake by β-alanine, hypotaurine and GABA. Uptake of 1 mM 5-ALA by WiDr cells was measured after 4 h of incubation in the presence of β-alanine (■), hypotaurine (●) or GABA (Δ) at concentrations as indicated in the figure. The cells were incubated in HEPES-buffer as described in the Materials and Methods section.

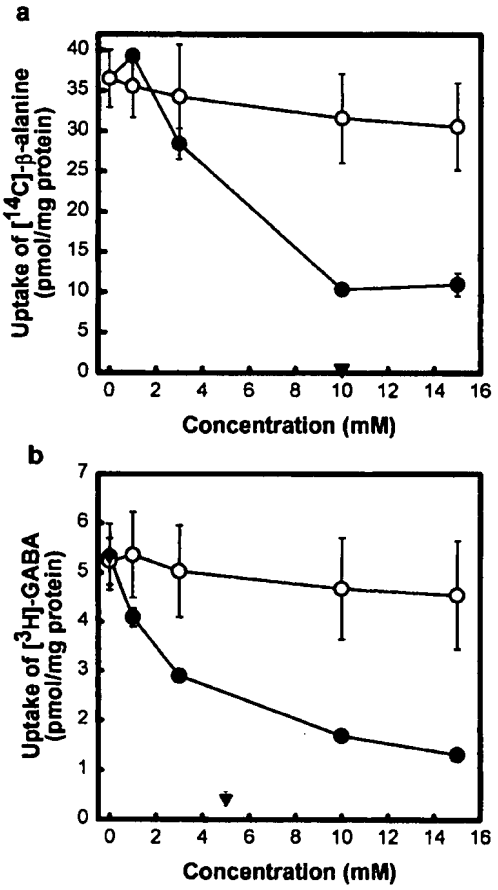

Figure 7. Influence of 5-ALA and 5-ALA methyl ester on uptake of β-alanine and GABA. Uptake of 5 μ*M* [^{14}C]-β-alanine (a) and 5 μ*M* [^{3}H]-GABA (b) was measured after 10 min of incubation in the presence of 5-ALA (●), 5-ALA methyl ester (○) or non-radioactively labeled β-alanine or GABA (▼) at concentrations as indicated in the figure.

DISCUSSION

The data presented herein indicate that transport of 5-ALA into human adenocarcinoma cells (WiDr cells) is mediated by an active transport mechanism. The uptake was found to be saturable following Michaelis–Menten kinetics (Fig. 1), with an activation energy that is within the range of active transport (Fig. 2). The transport was found to be Na^+- and Cl^--dependent and inhibited by several zwitterionic and basic amino acids, in particular the β-amino acids, β-alanine and taurine, as well as by GABA (Figs. 4 and 6–8). Furthermore, the transport has also recently been found to be energy-dependent (8). The transporter(s) involved display(s) a low affinity (K_m = 8–10 m*M*), but at the same time a relatively high capacity (V_{max} = 18–20 nmol·(mg protein × h)$^{-1}$) for the uptake of 5-ALA in WiDr cells.

In WiDr cells PpIX formation increases with extracellular 5-ALA concentrations up to about 1 m*M*, above which PpIX formation is independent of the 5-ALA concentration (6). It is demonstrated here that K_m for uptake of 5-ALA in WiDr cells, under similar conditions, is 8–10 m*M*. This means that at extracellular concentrations of 5-ALA above 1 m*M*, transport of 5-ALA over the plasma membrane is not the rate-limiting step in PpIX formation. Of the enzymes involved in the heme synthesis, porphobilinogen deaminase and coproporphyrinogen oxidase may be rate-limiting steps in the formation of PpIX (12–14). Thus, these enzymes may constitute the rate-limiting step in the formation of PpIX at extracellular concentrations of 5-ALA above 1 m*M*.

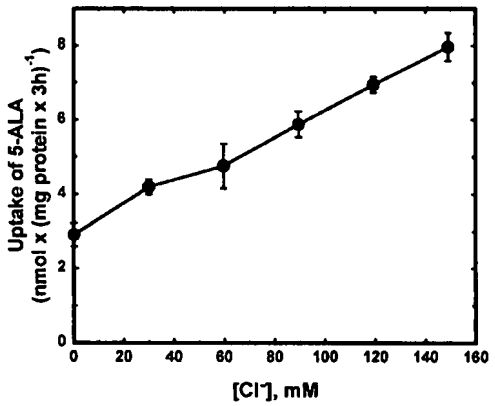

Figure 8. Dependence of 5-ALA uptake upon Cl^-concentration. WiDr cells were incubated with 1 m*M* 5-ALA in a HEPES-buffer in which the Cl^- concentration is varied by substituting NaCl with Na gluconate, $MgCl_2$ with $MgSO_4$ and $CaCl_2$ with $Ca(NO_3)_2{\cdot}4H_2O$, and otherwise treated as described in the Materials and Methods section. Uptake of 1 m*M* 5-ALA was measured after a 3 h incubation.

The calculated activation energy of 112 kJ·mol^{-1} for 5-ALA transport in the temperature range 15–32°C (Fig. 2b) clearly exceeds that anticipated for simple diffusion, and is in the range for carrier-mediated processes (E_a > 30 kJ·mol^{-1} (15)). Above 32°C the uptake of 5-ALA was less temperature-dependent and the activation energy was about 26 kJ·mol^{-1}. This is an activation energy which is higher than that for simple diffusion (E_a < 17 kJ·mol^{-1} (15)), but below the usual range for carrier-mediated processes. The observed discontinuity in the Arrhenius plot at 32°C may be interpreted as a phase transition in the membrane fluidity (16). The phase transition temperature varies with the lipid

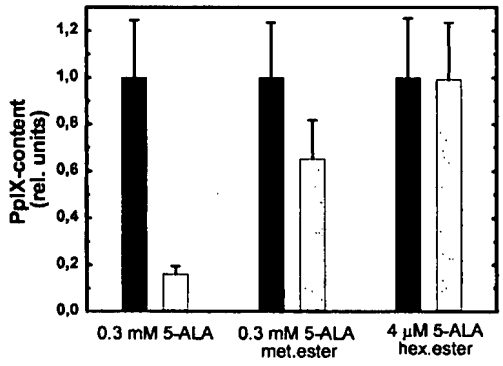

Figure 9. Effect of β-alanine upon the accumulation of PpIX in WiDr cells treated with 5-ALA or 5-ALA esters. The cells were treated with 0.3 m*M* 5-ALA, 0.3 m*M* 5-ALA methyl ester or 4 μ*M* 5-ALA hexyl ester for 4 h in RPMI 1640 medium without serum, in the absence (black bars) or presence (grey bars) of 10 m*M* β-alanine.

composition of the membrane, *e.g.* for alanine transport it varied between 16°C in CHO cells enriched in oleate and 32°C in cells enriched in palmitate (17).

Separate transport systems usually serve for cationic, zwitterionic and anionic amino acids in most mammalian cell types (18). According to the classification by Stevens *et al.* (19), carrier systems for amino acids have been categorized into Na^+-dependent carriers (systems B, A, ASC, N, glycine (Gly), X^-_{AG}, BETA, IMINO and GABA) and Na^+-independent carriers (systems L, y^+, $b^{0,+}$ and x^-_c). The transport of 5-ALA was both Na^+- and Cl^--dependent (Figs. 3 and 8). Several of the Na^+-dependent amino acid transporters are most likely not specific transporters for 5-ALA: (1) 5-ALA was recently found not to influence on the uptake of methyl-AIB, specifically transported by the widespread system A (8); (2) threonine, but not glycine, reacts well with system ASC, while glycine, but not threonine, is a competitive inhibitor for 5-ALA transport (20) (Fig. 4). System ASC is relatively pH-insensitive and *trans*-stimulated, which is not the case for transport of 5-ALA (8); (3) system Gly is specific for glycine and sarcosine, while the transporter(s) for 5-ALA has a broad specificity (21); (4) system X^-_{AG} has a specificity for glutamate and aspartate which do not significantly reduce the uptake of 5-ALA (Fig. 4) (22); (5) systems N and N^m are specific for glutamine, histidine and asparagine, which also are inhibitors of 5-ALA, but these transporters have only been found in liver and skeletal muscle (23); and (6) system IMINO does not seem to use neutral amino acids as substrates, which is in contrast to the 5-ALA transporter(s) (24). However, both the system BETA superfamily (transporting β-alanine, taurine and GABA) and system $B^{0,+}$, are dependent upon Na^+ and Cl^- (23,25–27), and are thus candidates for transport of 5-ALA. System $B^{0,+}$ has a broad specificity similar to that of the 5-ALA transporter, and it is also inhibited by β-alanine, but displays a lack of specificity for taurine at least in mouse blastocytes (28,29). The observed inhibition of 5-ALA transport by β-alanine, taurine (substrates for system BETA) and GABA (Figs. 4, 6 and 7), indicates a transport of 5-ALA through system BETA and the GABA transporters, which have overlapping substrate specificities (30,31). It was reported that transport of β-alanine, as found for 5-ALA in this study (Fig. 4), was inhibited by lysine, leucine, glycine, serine, alanine and proline in the epithelium from rabbit distal ileum (32). However, in the HT-29 human colon carcinoma cell line, transport of taurine was inhibited by β-alanine, but not by alanine and leucine (33). Brush-border membrane vesicles from proximal intestine contain a β-alanine transporter with high specificity (10). The uptake of β-alanine through this transporter was not inhibited by proline, lysine and aspartate. This is in contrast to the 5-ALA transport pointing towards tissue and species differences in transporter specificity. GABA transporters have been shown to be expressed in many tissues outside central nervous system (11,34–39). The GABA transporters GAT-2 and GAT-3 are inhibited 60 and 80%, respectively, in the absence of Cl^-, similar to the transport of 5-ALA (Fig. 8), while that of GAT-1 is 95% inhibited (11). Based on these considerations, it thus seems that the transport of 5-ALA may occur on both the β-amino acid and GABA transporters, while transport on system $B^{0,+}$ cannot be excluded. However, it should be emphasized that inhibition of β-alanine transporters could account for 85% of PpIX formed in cells treated with 0.3 m*M* 5-ALA (Fig. 9), pointing towards the importance of β-alanine transporters for the uptake of 5-ALA. Whether transporters of other metabolites like the apical peptide transporters PEPT1 and PEPT2, as found in epithelial cells of the intestine and kidney (53,54), are also involved in transport of 5-ALA is not known.

Neoplastic cells exhibit increased demands for certain metabolites, including amino acids, which must be transported across the plasma membrane. This results in not only an enhanced expression of amino acid transporters, but also in the expression of isoforms not found in their normal counterparts (40,41). In general, little is known about amino acid transport in human neoplastic cells, and comparison with transporters in normal cells must therefore be executed with caution. Colon carcinoma cell lines, particularly HT-29 and Caco-2, have been shown to exhibit enterocyte-like differentiation features (42). It has been documented that colon carcinoma cells (HT-29) express a common transporter for both taurine and β-alanine (33), indicating that a similar transporter may be involved in the transport of 5-ALA in the WiDr colon adenocarcinoma cells. There are so far no reports indicating GABA transporters in colon carcinomas, and the overlapping specificities of β-amino acid and GABA transporters suggest that GABA competes with 5-ALA for transport through a β-amino acid transporter in WiDr cells. However, the results from this study indicate that transport of 5-ALA by GABA transporters in other cell types should be considered. Previous reports indicating that 5-ALA is transported by the GABA transporter, UGA4, in *Saccharomyces cerevisiae* support such a notion (43). An enhanced expression of amino acid transporters may also account for accumulation of more PpIX in neoplastic tissues treated with 5-ALA than in their normal counterparts (2).

Systemic administration of 5-ALA to patients induces a significant decrease in systolic and diastolic blood pressure (44). GABA regulates cardiovascular function *via* central and peripheral GABA receptors (45–47). Intravenous administration of GABA lowers blood pressure and induces bradycardia (48). Thus, the 5-ALA–induced lowering of blood pressure may be related to its binding to GABA receptors (49). Furthermore, GABA transporters are found in peripheral neurons (50–52). Thus, 5-ALA, but not 5-ALA esters (Figs. 6, 7 and 9), should be expected to be preferentially taken up into peripheral nerve endings. This may explain the clinical experience with the more-severe pain reactions observed during light exposure of basal cell carcinoma lesions after topical application of 5-ALA as compared to 5-ALA methyl ester (A. Soler, personal communication). The lack of inhibitory effect of β-alanine upon 5-ALA ester–induced PpIX formation (Fig. 9) and lack of inhibitory effect of 5-ALA ester on the transport of 5-ALA (Fig. 4), indicate that 5-ALA esters are taken up by transporters other than those used for uptake of 5-ALA or that they may penetrate the plasma membrane by passive diffusion. Different transport mechanisms between 5-ALA and 5-ALA-esters may provide a molecular explanation for some of the differences observed with such compounds in clinical PDT.

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